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Impact of Roundup Ready® Canola on Plant Pathogen and Its Suitability as Animal Feed: A Case Study in Canada

H. C. Huang, R. Sharma, and R. E. Blackshaw

Agriculture and Agri-Food Canada, Research Centre, Lethbridge, Alberta, Canada
Corresponding author, e-mail: huangh@agr.gc.ca

ABSTRACT

The steady increase in world production of genetically modified (GM) or transgenic crops since their introduction in the 1990s has caused some concerns, regarding the bio-safety on persistence and stability of recombinant DNA from GM crops. A field experiment was established in 2000 at the Agriculture and Agri-Food Canada Lethbridge Research Centre to investigate the long-term environmental impacts of some GM crops approved for production in Canada, including GM canola, corn and potatoes. One of the objectives of this study was to investigate horizontal gene transfer (HGT) from Roundup Ready® (RR) canola (*Brassica napus* event RT73) to *Sclerotinia sclerotiorum*, causal agent of stem blight of canola. The investigations focused on the *cp4 5-enolpyrulylshikimate-3-phosphate synthase* (*cp4 EPSPS*) gene, which endows RR canola with herbicide tolerance towards glyphosate. PCR and Southern hybridization were used to test sclerotia of *S. sclerotiorum* produced in diseased stems of RR canola and conventional canola, which were collected from the field in 2004 and 2005, for the presence of the 1363 bp *cp4 EPSPS* transgene and four of its fragments; three of which were construct-specific and one that was gene-specific. Results of PCR showed that *cp4 EPSPS* was present in diseased RR canola

stems but was absent in sclerotia of *S. sclerotiorum* formed inside the same stems. These results were further confirmed by Southern blotting and hybridization. The study concludes that there is no evidence for recombinant DNA transfer of *cp4 EPSPS* or any of its constituent fragments from RR canola to the pathogen *S. sclerotiorum*. From our studies with animals we found that feed-ingested DNA fragments do survive the terminal Gastrointestinal (GI) tract, but recombinant DNA in the gut is processed similar to any other endogenous feed-ingested genetic material. Plant DNA from GM feed degrades very rapidly upon its release into the rumen fluid. We did not detect any *cp4 EPSPS* fragments in the microbial DNA fraction of *in vitro* ruminal cultures, suggesting that bacterial transformation did not occur. Based on our investigations it was clear that uptake of transgenic DNA fragments by ruminal bacteria is precluded or time-limited by rapid degradation of plant DNA upon plant cell lysis. Thus use of GM canola as animal feed is safe and should be encouraged based on the scientific findings.

Key Words: Bio-safety; cp4 EPSPS gene; Sclerotia; *Sclerotinia sclerotiorum*; Canola; *Brassica napus*; Horizontal gene transfer; Roundup Ready® canola; Glyphosate.

INTRODUCTION

Canada was the fourth world largest country in the world for commercial production of genetically modified (GM) crops in 2005 with canola, corn and soybean as the major GM crops (James, 2005). The GM canola crops, Roundup Ready® (glyphosate tolerant) (RR canola) and Liberty Link® (glufosinolate tolerant) (LL canola), were first registered in Canada in 1995 (Stringham *et al.*, 2003). They constitute over 75% of all canola production in Canada (Canola Council of Canada, 2001; Stringham *et al.*, 2003). In 2003, the area for RR canola was 3.2 million ha in

Canada (Traxler, 2006). The estimated economic benefits of RR canola in Canada in 2000 were about $47 million (29%) accruing to producers, $93 million (57%) accruing to industry, and $21 million (14%) accruing to consumers (Philips, 2003). Consumer acceptance of GM canola has generally been very good in Canada and its trading partners, except in Europe where there is a ban on the use of GM canola and its products (Stringham *et al.*, 2003).

Despite the overall benefits of growing transgenic crops, there are several concerns regarding their ecological and environmental biosafety. These include higher reported levels of *S. sclerotiorum* infection in glyphosate tolerant crops (Lee *et al.*, 2003); evolution of herbicide resistant weeds (Llewellyn *et al.*, 2002; Owen and Zelaya, 2005); recombinant DNA movement within and across species; and persistence, stability and potential uptake of the transgenic DNA by viruses, bacteria, plant, animal and fungal cells (Ho *et al.*, 2000a; 2000b; Fincham *et al.*, 1989).

Horizontal gene transfer (HGT) has been implicated in the evolution of fungi (Rosewich and Kistler, 2000) but the exact mechanism(s) and reason behind such an event has not been clearly understood. *Sclerotinia sclerotiorum* is a fungal pathogen which often produces black sclerotia on diseased canola plants. Such intimate host-pathogen relationships make this pathogen an ideal candidate for studying horizontal gene transfer from GM canola to the pathogen. Since canola meal is an important constituent of animal diets, both monogastrics and ruminants, it is pertinent to assess the fate of the recombinant DNA arising from GM canola when used as animal feed.

A long-term (12 years or longer) field study was initiated in 2000 at the Agriculture and Agri-Food Canada Research Centre in Lethbridge, Alberta, to assess the

environmental and economic impact of GM crop production in Canada. Specific objectives of this study were to determine: 1) population dynamics of weeds, diseases and insects (of target and non-target species), 2) soil microbial diversity, 3) herbicide resistance development, 4) gene flow, 5) crop yield and quality, and 6) economic opportunity and risk to the farmer. The objectives of this report will focus the discussion only in two of these areas: the impact of RR canola on the pathogen *Sclerotinia sclerotiorum* and the suitability of RR canola as animal feed.

RESULT AND DISCUSSION

Design of the Field Experiment of GM Crops

The field experiment was established in 2000 in a 10-ha area at the Lethbridge Research Centre, Agriculture and Agri-Food Canada, to investigate the long-term environmental impacts of some GM crops approved for production in Canada, including RR canola, LL canola, RR maize, LL maize, *Bt* maize and *Bt* potato. The experiment consisted of 10 treatments with four replicates arranged in a randomized complete block design. The individual plot size was 15 m x 35 m (W x L). There were 3 m pathways between plots and 20 m pathways between replicates to maintain treatment integrity over many years.

Diseases of GM Canola and Conventional Canola in the Field (2000-2005)

When canola plants in plots of RR canola, LL canola and conventional canola (CC) reached maturity stage (mid-August) in each year, they were examined for natural occurrence of diseases. Plants were rated for each disease by inspecting five sites in each plot, with 100 plants (50 per row) in two rows per site. Sites were located in an

X pattern, with 5 m separation between sites. At each site, the number of plants with symptoms for each disease was recorded.

Results of disease survey showed that no diseases were observed in all canola (RR canola, LL canola and CC canola) in the first year (2000). During 2001-2005, three diseases were detected, including sclerotinia stem rot *(Sclerotinia sclerotiorum)* (Table 1), staghead (*Albugo candida*) (Table 2), and alternaria pod spot (*Alternaria brassicae* and *A. raphani*) (Table 3). However, incidence of these diseases was low (<7%) and, for each disease, there was no significant differences among the treatments of RR canola, LL canola and CC canola (Tables 1-3). Incidence of alternaria pod spot was 16.8% in CC canola in 2002 and 16.2% in RR canola in 2004 (Table 3). This high incidence of alternaria pod spot was associated with damage from a flea beetle infestation.

Table 1. Incidence of Sclerotinia rot (*Scleroitnia sclerotiorum*) in Roundup Ready® canola, Liberty Link® canola and Conventional canola.

	Sclerotinia Disease Incidence (%)					
Treatment	2000	2001	2002	2003	2004	2005
Conventional	0.0	0.7	0.0	0.1	1.6	2.9a
Liberty Link®	0.0	0.3	0.0	0.4	0.4	3.2a
Roundup Ready®	0.0	0.3	0.0	0.1	1.3	2.8a

Table 2. Incidence of staghead (*Albugo candida*) in Roundup Ready® canola, Liberty Link® canola and Conventional canola.

	Staghead Disease Incidence (%)					
Treatment	2000	2001	2002	2003	2004	2005
Conventional	0.0	0.6	0.0	0.0	0.3	0.3
Liberty Link®	0.0	0.3	0.0	0.0	0.2	0.2
Roundup Ready®	0.0	0.2	0.0	0.0	0.2	0.4

Table 3. Incidence of alternaria pod spot (*Alternaria brassicae* and *A. raphani*) in Roundup Ready® canola, Liberty Link® canola and Conventional canola.

	Alternaria Disease Incidence (%)					
Treatment	2000	2001	2002	2003	2004	2005
Conventional	0.0	0.2	16.8*	6.1	4.0	2.0
Liberty Link®	0.0	0.2	2.3	3.1	2.1	2.2
Roundup Ready®	0.0	0.1	3.8	6.5	16.2*	2.6

* Alternaria infection was associated with flea beetle damage

Assessment of Horizontal Gene Transfer from RR Canola to *Sclerotinia sclerotiorum*

Samples of canola stems and sclerotia of *S. sclerotiorum* collected in 2004 and 2005 were used in this study. In each year, canola stems infected by *S. sclerotiorum* and sclerotia produced inside the stems were collected from plots of RR and CC canola in early September. Healthy stems of canola from these plots as well as sclerotia of *S. sclerotiorum* produced on diseased bean plants in another field were also collected for use as controls. Sclerotia were washed in running water for 10 minutes in a sieve,

and rubbed between two layers of paper towel to remove all the remaining plant material from the sclerotial surface. The stems and sclerotia were then used for testing presence of the RR-gene by PCR analyses and Southern hybridization (Sharma *et al.*, submitted) (Table 4).

Table 4. Detection of *cp4 EPSPS* and its fragments in *Sclerotinia sclerotiorum,* ruminal cultures and animal digesta/tissues.

Detection of *cp4 EPSPS*	Duration of the Study	Findings	Reference
Sclerotinia sclerotiorum	2 crop seasons	Negative	Sharma *et al.* (submitted)
Ruminal *in vitro* cultures	48 h	Transgene fragments detected up to 48 h; no transformation of ruminal bacteria	Sharma *et al.*, 2004
Ruminal fluid	4 h	Detected up to 10 min	Alexander *et al.*, 2004
Animal GI* tract contents	Feeding trial (3-4 months)	Detected in digesta samples of lambs and swine.	Sharma *et al.*, 2006
Animal GI* tract tissues	Feeding trial (3-4 months)	Detected at a low frequency from lambs and swine	Sharma *et al.*, 2006

*GI, gastrointestinal tract

PCR analysis of conventional and Roundup Ready® canola stems for the 1363 bp *cp4 EPSPS* transgene confirmed the GM trait of the canola plants from which the sclerotia samples were derived; all the CC stems showed absence of 1.3 kb transgene whereas all the RR stems tested positive (Fig.1). PCR assays for the 1363 bp *cp4 EPSPS* did not yield positive results for any of the samples of sclerotia of *S. sclerotiorum* collected from plots of RR canola in 2004 and 2005 (Fig. 2A). Representative gel for the non-detection of three different construct- or gene-specific fragments (F1, F3, F4) ranging in size from 278 to 527-bp are also shown in Fig. 2

(B, C, D). The non-detection of 1363 bp *cp4 EPSPS* is not surprising since previous study using *in vitro* culture indicates that the complete transgene degrades rapidly depending on the extent of processing of the sample (Alexander *et al.*, 2002). Thus, the possibility of gene transfer of *S. sclerotiorum* is low despite the intimate association between GM canola and the pathogen due to the well-known active mechanisms of degradation and excision of foreign DNA.

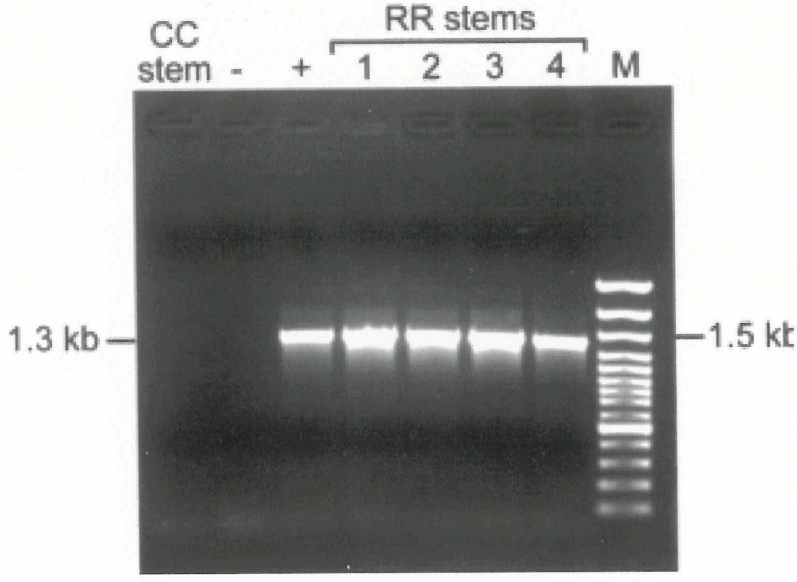

Fig.1. PCR analysis of 1363 bp *cp4 EPSPS* from diseased Roundup Ready® canola stems. Lane CC: conventional canola lacking the transgene (negative extraction control); Lane -: negative PCR control (no DNA template); Lane +: positive control (Roundup Ready® leaf DNA as template); Lanes 1-4: PCR product from four diseased stems.

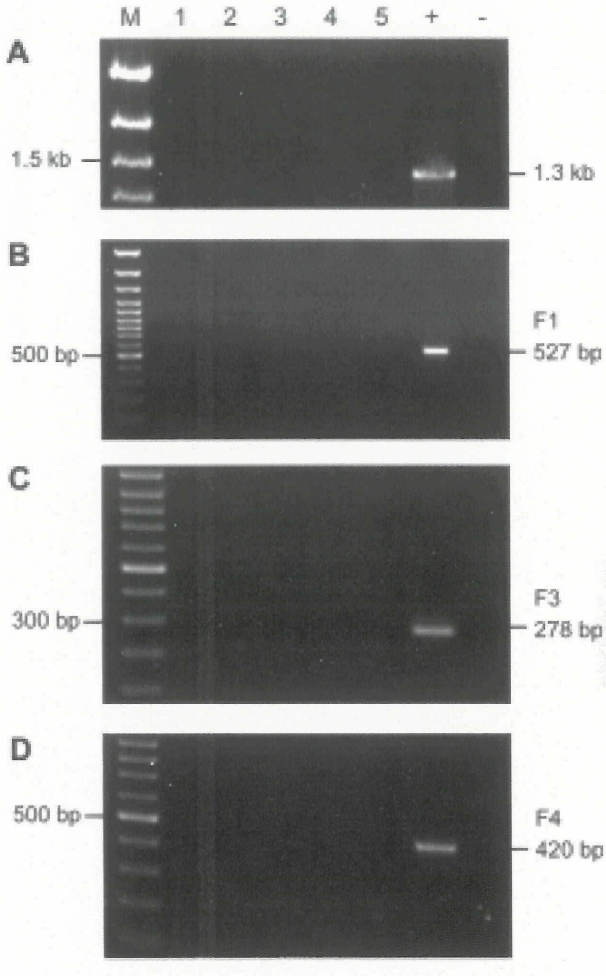

Fig. 2. Representative gel for PCR amplification of *cp4 EPSPS* and fragments F1, F3, F4 from sclerotia collected from diseased Roundup Ready® stems. A: absence of the 1363 bp *cp4 EPSPS*. Lanes 1, 2, 4, and 5: PCR products from four different sclerotia. Lane 3: blank (no PCR product loaded). B: Absence of fragment F1 from sclerotia. Lanes 1-5: PCR products from five different sclerotia. C: Absence of fragment F3 from sclerotia. Lanes 1-4: PCR products from four different sclerotia. Lane 5: blank. D: Absence of fragment F4 from sclerotia samples. Lanes 1-5: PCR products from five different sclerotia. For all the panels Lane M: 100 bp DNA ladder; Lane +: positive control (Roundup Ready® leaf DNA as template); Lane - : negative control (no DNA template).

Effects of RR Canola on Ruminant Animals and Ruminal Bacteria

We evaluated Roundup Ready® canola meal in barley based diets for lambs and found that digestibility, feed efficiency, growth performance, carcass characteristics or meat quality of lambs fed the transgenic diet was no different than the animals fed conventional canola diets (Stanford *et al.*, 2003). While assessing the fate of recombinant DNA in mixed ruminal cultures we observed that upon processing of canola, DNA large enough to contain intact plant gene remains unlike the report by Chiter *et al.* (2000) where treatment of canola meal resulted in complete degradation of DNA and heating maize grains to 95° C for 5 min resulted in an inability to PCR amplify a 577-bp sequence. In mixed ruminal cultures, plant DNA was found to be rapidly degraded upon its release into the rumen fluid. However we could detect *cp4 EPSPS* (1363 bp) up to 8 h from meals and 4 h from mixed diets (Alexander *et al.*, 2002). Research from our laboratory provided a comparison for the first time on the stability of complete transgene versus the smaller transgene fragments. We found that detection of fragments was representative of persistence of the whole transgene (Sharma *et al.*, 2004) and that no transformation was observed in the ruminal bacteria up to at least 48 h. Transformation of gut micro-organisms with the recombinant DNA does not seem possible given the high nuclease activity (i.e. enzymes that degrade DNA or RNA) in the rumen and the gastrointestinal tract. It is likely that the plant DNA released in the digestive tract is degraded into multitude of smaller fragments of different sizes which may exhibit differential stability. However, if these fragments are associated with the undigested feed particles, these are excreted via feces.

Using extremely low limit of detection of the PCR assay (12.5 pg), we elucidated that no *EPSPS* fragments are amplifiable in microbial fraction of the ruminal cultures suggesting that transformation had not occurred during 48 h incubation. Further our

results indicate that uptake of transgenic DNA fragments by ruminal bacteria is probably precluded or time-limited by rapid degradation of plant DNA-transgenic or otherwise upon cell lysis (Sharma *et al.*, 2004). Using quantitative real-time PCR we found that free DNA is rapidly degraded at neutral pH of duodenal fluid reducing the likelihood of transgenic DNA uptake in the Gastrointestinal (GI) tract. The *cp4 EPSPS* gene and four of its fragments were detected from ruminal fluid up to only 10 min, however these could be detected from the feces up to 2 - 4 h (Alexander *et al.*, 2004).

In a feeding trial conducted using sheep (n=11) and pigs (n=36) containing 6.5% and 15% Roundup Ready® canola, respectively, the transgene was tracked in digesta and GI tract tissues. We found low-copy *ribulose-1,5-bisphosphate carboxylase /oxygenase* (*rubisco;*186- and 540-bp) fragments were present at slightly lower, variable frequencies in digesta (18-82%) and intestinal tissues (9-27% of ovine and 17-25% of porcine samples), and infrequently in visceral organs (1 of 88 ovine samples; 3 of 216 porcine samples). Each of the five *cp4 EPSPS* transgene fragments (179 to 527 bp) surveyed were present in at least 27% of ovine large intestinal content samples (max. 64%) and at least 33% of porcine cecal contents samples (max. 75%). We found that transgenic DNA has the same fate as endogenous plant DNA when used as animal feed.

Effect of RR Canola on Weed Community Dynamics

Weed density by species was determined 1) before seeding, 2) before in-crop herbicide application, and 3) before crop harvest. Weed counts were made in fifteen quadrats (0.25 m^2) per plot utilizing a 'W' pattern. The weed seed bank was determined at the beginning of the experiment and it will be determined again at the

conclusion of the study. Visual assessments of potential herbicide resistant plants were made throughout the growing season in each year.

Preliminary results showed that weed densities were lower in both RR canola and LL canola compared to conventional canola (CC canola). This usually resulted in higher canola yield (R. E. Blackshaw *et al.* unpublished data). No weeds resistant to glyphosate (Roundup herbicide) or glufosinate (Liberty herbicide) were found in this experiment or anywhere in Canada.

CONCLUSIONS

The field study at the Agriculture and Agri-Food Canada Research Centre in Lethbridge, Alberta, reveals that the transgene *cp4 EPSPS* for tolerance to glyphosate in RR canola is incapable of spreading onto *S. sclerotiorum*, despite the intimate host-parasite relationship. There is no significant difference in occurrence of diseases between GM canola and CC canola as the incidence for each of the three diseases remains low during the period of 2000-2005.

Regarding the use of Roundup Ready® canola as animal feed we found that transgenic DNA behaves no differently than plant endogenous DNA. We did not find any uptake of transgene or its fragments into the ruminal microbial population under the period of time investigated. The transgenic DNA could be detected from the digesta in the GI tract at variable frequencies in lambs and swine fed the transgenic diets.

The trend toward an increase in production of transgenic crops will likely continue around the world. The negative findings on horizontal gene transfer from GM canola to *S. sclerotiorum*, ruminal bacteria, and fecal material of sheep and pig at the Lethbridge Research Centre in Canada support the view of Traxler (2006) that the environmental effects of transgenic crops have been strongly positive to date. Nevertheless, the long-term environmental concerns of GM crops or GM organisms deserve continued monitoring, because of the novel nature of the GM technology. This include long-term studies to determine whether gene flow from RR canola to related plant species will have a negative effect on biodiversity or on control of weeds.

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